

**Radiation of endemic Hawaiian 'opihi (*Cellana* spp.)**

Ashlee Albright

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Department of Biology

Hawaii Institute of Marine Biology

Advisors: Chris Bird, Brian Bowen, Robert Toonen and Brenden Holland

## **Abstract**

Three endemic Hawaiian limpets (*Cellana exarata*, *C. sandwicensis* and *C. talcosa*), known locally as 'opihi, inhabit the wave-exposed rocky shores of the Hawaiian Archipelago. In a previous study using phylogenetic evidence derived from mitochondrial 16S ribosomal RNA, the ancestors of 'opihi were determined to have colonized the archipelago in two separate events. The objective of this study was to use multiple loci, including both nuclear and mitochondrial markers, to illuminate the phylogenetic history of the Hawaiian 'opihi. Samples were collected from various locations throughout the Indo-Pacific. Genomic DNA was extracted and the polymerase chain reaction was used to amplify 16S ribosomal RNA (16S), cytochrome *c* oxidase subunit I (COI), histone (H3) and ATP-synthetase beta-subunit (ATPS- $\beta$ ) genes. Sequences were analyzed using PAUP\* to yield parsimony, distance and maximum likelihood phylogenetic trees. The inferred preliminary phylogeny indicates that the Hawaiian *Cellana* lineage is monophyletic based on combined COI, H3 and ATPS- $\beta$  reconstructions, contradicting the 16S mtDNA phylogeny. This preliminary result suggests a single colonization event followed by subsequent diversification – the first such event to be described for a marine lineage within the Hawaiian Archipelago.

## **Introduction**

The Hawaiian Archipelago is a unique environment wherein there have been no reports of major marine faunal radiations comparable to those of terrestrial plants and animals (Hourigan and Reese 1987, Kay and Palumbi 1987). This may be attributed to the dispersal potential of Hawaiian marine life and to a number of island characteristics such as geographic isolation and current patterns (Hourigan and Reese 1987). Kay (1980)

singles out a species assemblage of endemic Hawaiian limpets (*Cellana* spp.), known locally as ‘opihi, as a possible example of adaptive radiation among marine mollusks.

‘Opihi were traditionally considered an important source of protein for native Hawaiians and continue to be marketed locally as a delicacy (Kay and Magruder 1977, Kay *et al.* 1982, Rogers 1967). Over-harvesting in the main Hawaiian Islands (MHI) has been associated with dramatic declines in the size and abundance of wild individuals and in market-catch (Kay and Magruder 1977). Development of Marine Protected Areas (MPA) for these culturally significant mollusks requires an extensive understanding of their phylogenetic history and patterns of population connectivity (Bird *et al. In press*).

Three species of ‘opihi occupy wave-exposed rocky shores of the Hawaiian Archipelago (*Cellana talcosa*, *C. exarata* and *C. sandwicensis*), each inhabiting a distinct zone within the basalt shoreline. *C. talcosa* inhabits a subtidal range from the calcareous algal zone up to 10 m depths. *C. exarata* (black foot ‘opihi) is found higher within the intertidal zone than *C. sandwicensis* (yellow foot ‘opihi) (Kay and Magruder 1977, Kay *et al.* 1982).

Reeb (1995) proposes that the narrow range of species-specific habitat zonation among the Hawaiian ‘opihi has led to their lineage’s inability to differentiate beyond the three recognized species. Furthermore, cross-fertilization experiments between *C. exarata*, *C. talcosa*, and *C. sandwicensis* individuals have created viable hybrids (Bird pers. commun). An investigation into the phylogenetic history of Hawaiian ‘opihi is fundamental in understanding how the three species arose and how they continue to remain genetically distinct.

Reeb (1995) suggests that Hawaiian 'opihi are derived from two colonization events from the West Pacific. Evidence for this conclusion is based on a phylogeny of Pacific *Cellana* for the mitochondrial 16S ribosomal RNA (16S) locus (Reeb 1995). Reeb's phylogeny using the 16S locus shows that the Hawaiian 'opihi are paraphyletic, sharing a clade with *Cellana mazatlandica* from the Ogasawara Islands. An alternative explanation of this phylogeny, not mentioned in Reeb's dissertation is that there was a single colonization of *Cellana* to Hawaii, followed by diversification into the Hawaiian species we see today and with *C. mazatlandica* leaving the archipelago to colonize the Ogasawara Islands. Some limitations to Reeb's conclusions based on her 16S phylogeny are as follows:

1) a single gene was used for phylogenetic analyses 2) a small fragment (270 bp) of this gene was used 3) the taxa groupings in the Hawaiian *Cellana/C. mazatlandica* clade are represented by low bootstrap values (36 and 40).

Maddison and Knowles (2006) conclude that more accurate phylogenetic trees are constructed using sequence data from multiple loci for a given sample size. The objective of this study is to use multiple loci, including both nuclear and mitochondrial markers, to illuminate the phylogenetic history of the Hawaiian 'opihi.

## **Methods**

### *Sample Collection*

*Cellana* specimens were collected from various locations throughout the Indo-Pacific. When whole animals were not collected, small segments of mantle tissue were removed using a sterile razor blade. Tissue samples were then preserved by freezing or by submergence in 95% ethanol.

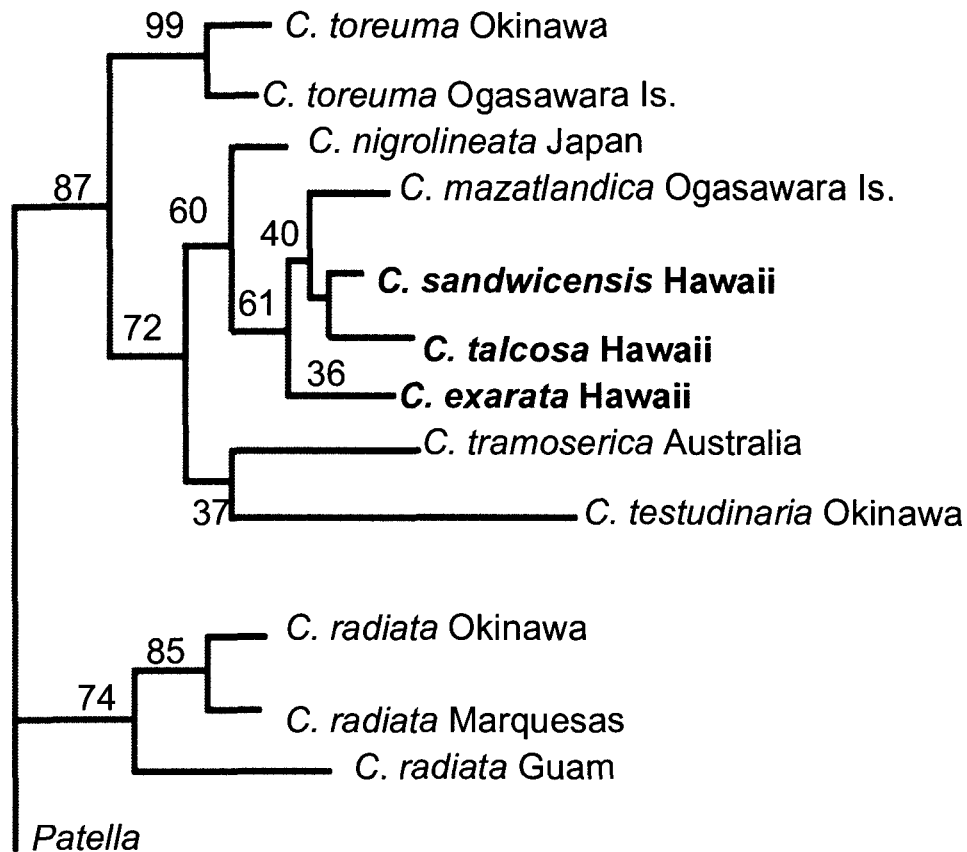
### *DNA Extraction, PCR, Sequencing and Sequence Analysis*

Qiagen (Qiagen Inc. Valencia, CA) DNeasy Animal Tissue Kits were used to extract genomic DNA from the collected tissue samples. The polymerase chain reaction (PCR) was used to amplify mitochondrial (cytochrome *c* oxidase subunit I (COI) and 16S ribosomal RNA) and nuclear (histone (H3) and ATP-synthetase beta-subunit (ATPS- $\beta$ )) loci. Addition of exonuclease 1 and shrimp alkaline phosphatase (exo-sap) to PCR products and immediate incubation in thermocyclers yielded purified PCR product for sequencing. DNA sequences were then aligned by eye and formatted into NEXUS files that were analyzed with PAUP\* to construct parsimony, distance and maximum likelihood phylogenetic trees.

Model Test was run for distance and maximum likelihood analyses and the best-fit models were selected to create each phylogenetic reconstruction. The phylogenies based on each of the four loci were created using Heuristic searches and 100 bootstrap replicate analyses were performed. In addition to the four phylogenies based on the separate loci, a concatenated phylogeny was created by joining individual sequences from all four markers into a single continuous sequence. One sample haplotype was selected for each of six species (*Cellana nigrolineata*, *C. grata*, *C. mazatlandica*, *C. exarata*, *C. sandwicensis*, *C. talcosa*) to create the concatenated phylogeny. In addition, an Exhaustive search, the TVM+I best fit model and 5000 bootstrap replicate analyses were used to create this tree.

### **Results**

Figure 1 displays Reeb's *Cellana* phylogeny, based on the mitochondrial 16S ribosomal locus. Note that the Hawaiian 'opihi are paraphyletic, sharing a clade with

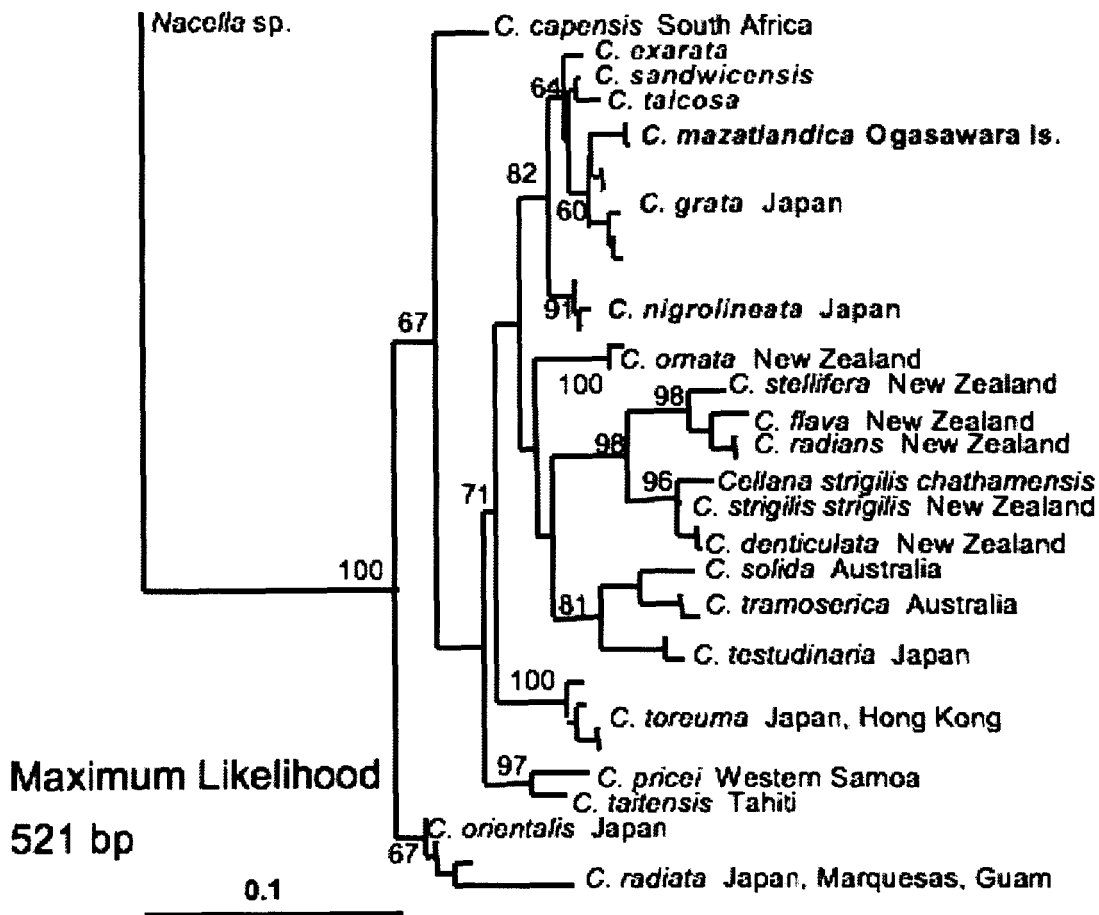


Treelength = 211  
270 bp

**Figure 1.** Unweighted parsimony consensus tree of Pacific *Cellana* for 16S MtDNA. Data set is bootstrapped 100 times and values are noted. Trees rooted with *Patella*. (Reeb 1995).

*Cellana mazatlandica*, from the Ogasawara Islands. The bootstrap values (36, 40) within this clade are low (ie. below 60, which is the lowest bootstrap value limit that is regarded as strongly supportive evidence for statistically reliable taxa groupings). Also note that the total length of the 16S gene fragment used is 270 base pairs.

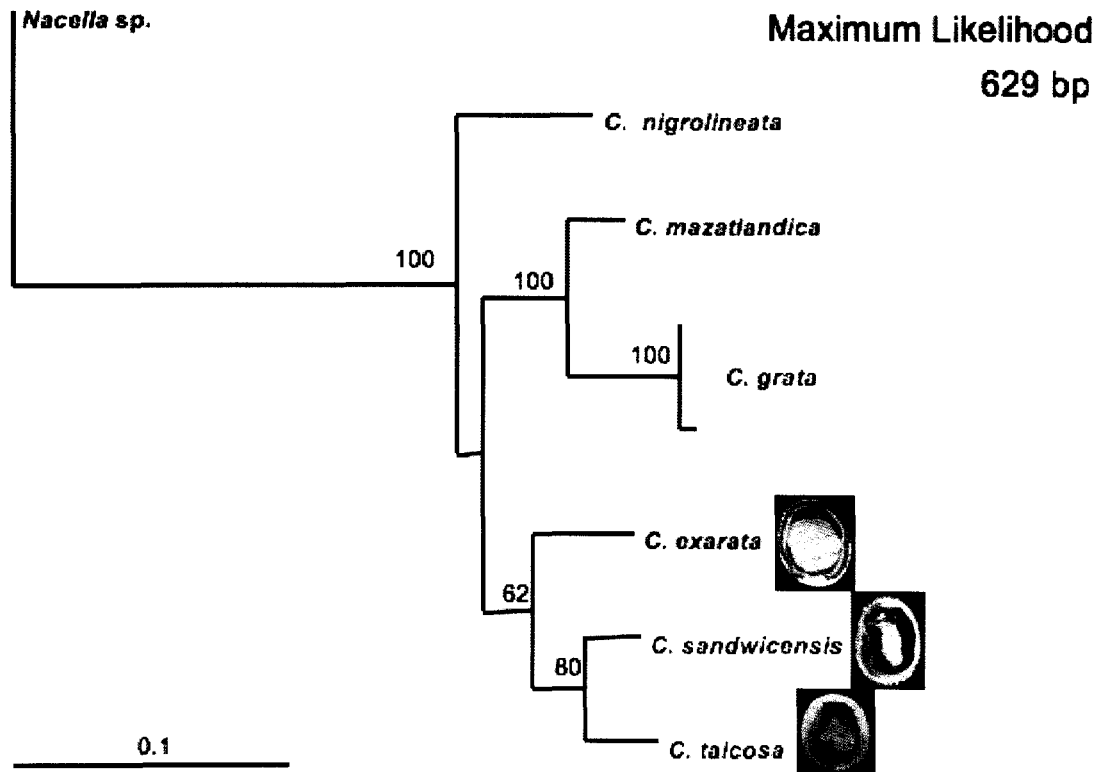
Figure 2 displays are similar phylogeny to Reeb's 16S mitochondrial tree in figure 1, however, this tree is based on a larger fragment (521 base pairs) of the 16S gene. It



**Figure 2.** Maximum Likelihood phylogeny based on a 521 base pair fragment of the 16S mitochondrial ribosomal RNA marker. Data set is bootstrapped 100 times and values are noted. Tree is rooted with *Nacella* sp.

shows, like Reeb's phylogeny, that the Hawaiian *Cellana* are paraphyletic and are closely related to Japan/Ogasawara congeners. Note, however, the larger bootstrap values (all above 60) at all branch nodes as opposed to Reeb's phylogeny. To date, this is the most comprehensive *Cellana* phylogeny using the 16S ribosomal locus. It incorporates sequence data from 22 species of *Cellana* sampled throughout the Indo-Pacific.

In this study, another mitochondrial marker, cytochrome *c* oxidase subunit I (COI), was used to create a phylogenetic reconstruction of *Cellana* spp. This phylogeny is shown in figure 3. Phylogenetic reconstructions based on two nuclear markers, histone

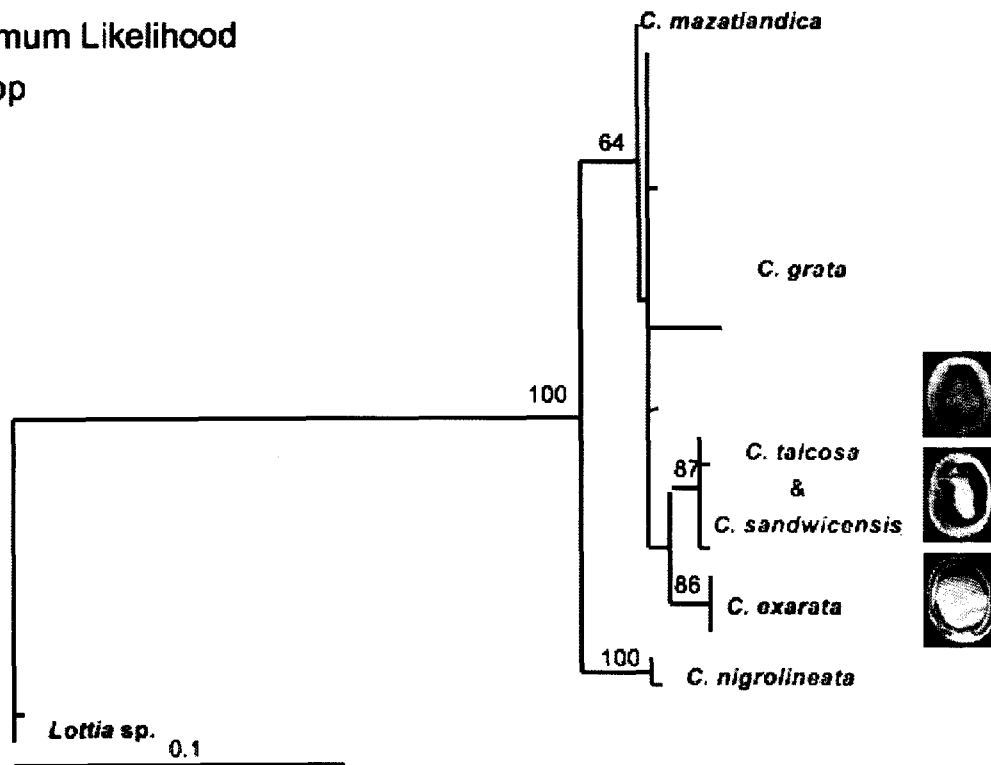


**Figure 3.** Maximum Likelihood phylogeny based on a 629 base pair fragment of the mitochondrial cytochrome *c* oxidase subunit I (COI) marker. Data is bootstrapped 100 times and values are noted. Tree is rooted with *Nacella* sp.

(H3) and ATP-synthetase beta-subunit (ATPS- $\beta$ ) are displayed in figures 4 and 5, respectively. For these three phylogenetic trees, note three reoccurring trends. First, the Hawaiian *Cellana* are monophyletic. Second, they are closely related to Japan and Ogasawara congeners. Finally, *C. talcosa* and *C. sandwicensis* are consistently grouped as sister-taxa.

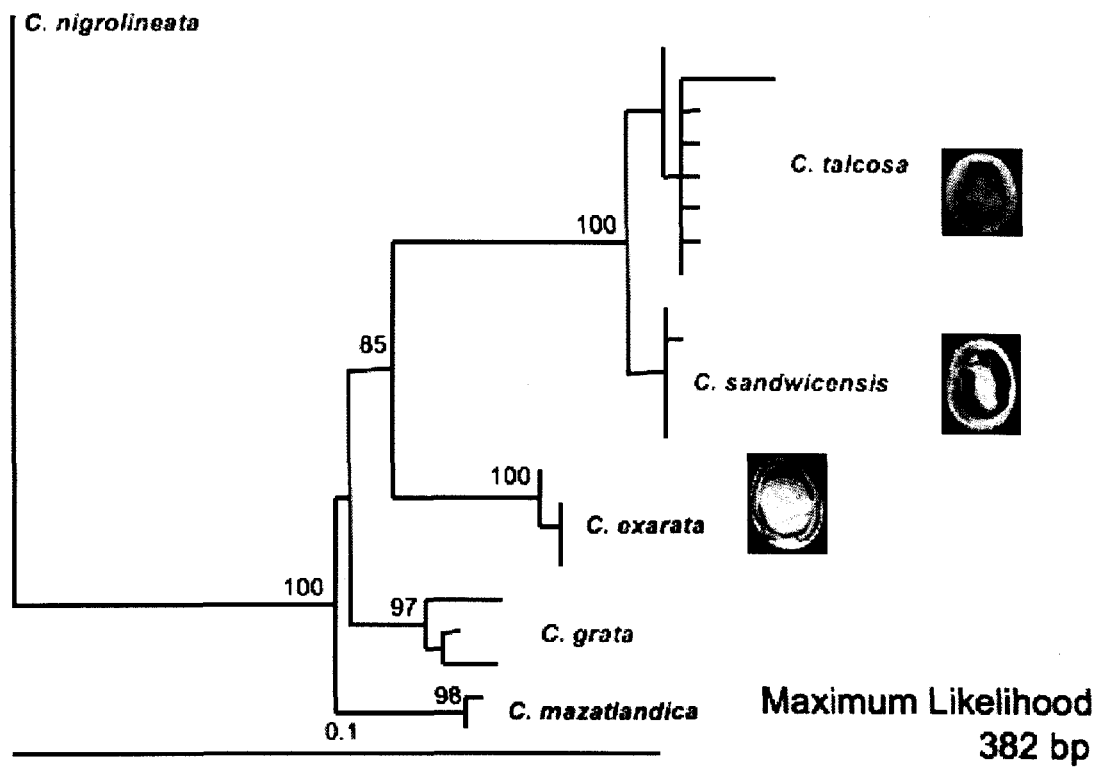


Maximum Likelihood  
328 bp

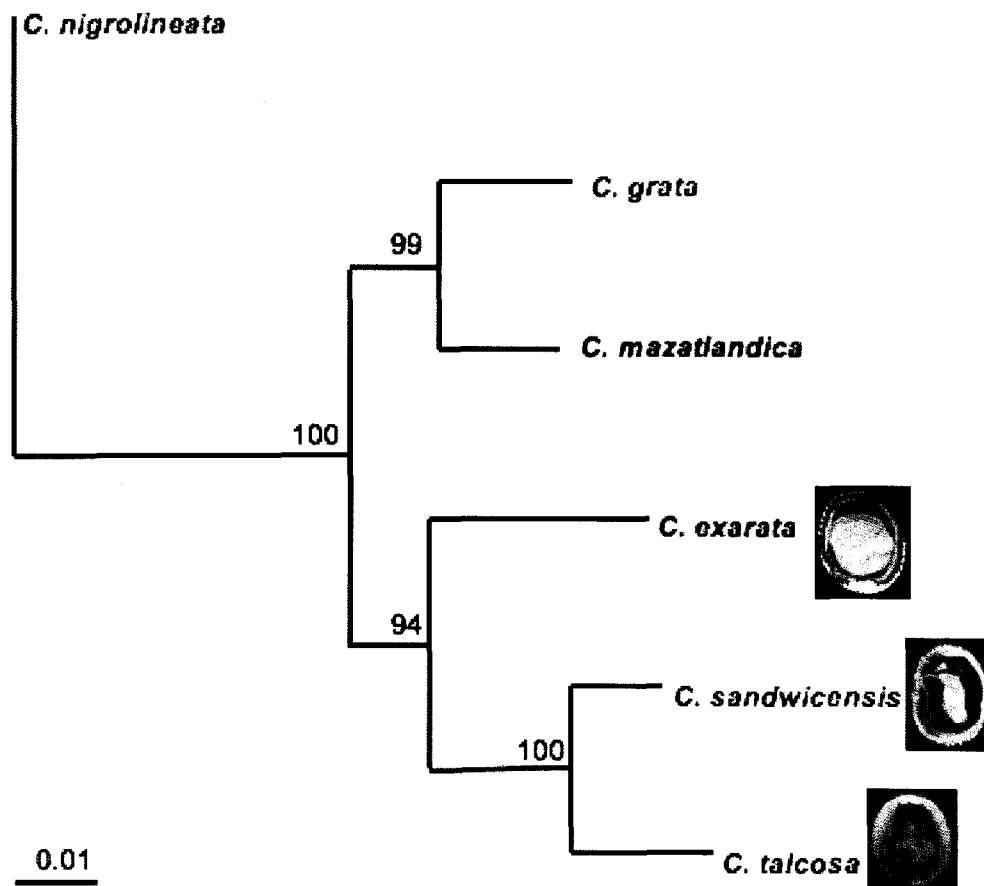


**Figure 4.** Maximum Likelihood phylogeny based on a 328 base pair fragment of the nuclear histone (H3) marker. Data is bootstrapped 100 times and values are noted. Tree is rooted with *Lottia sp.*

Combining the sequence data for all four genetic markers (16S, COI, H3 and ATPS- $\beta$ ) yields a concatenated phylogeny, illustrated in figure 6. This phylogeny incorporates 1860 base pairs of sequence data, thus, providing solid evidence for Hawaiian *Cellana* monophyly. It reaffirms their close relationship to Japan and Ogasawara Island congeners and shows *C. talcosa* and *C. sandwicensis* as sister-taxa. Note the high bootstrap values, all of which are greater than 94.



**Figure 5.** Maximum Likelihood phylogeny based on a 382 base pair fragment of the nuclear ATP-synthetase beta-subunit (ATPS- $\beta$ ) marker. Data is bootstrapped 100 times and values are noted. Tree is rooted with *C. nigrolineata*.



**Figure 6.** Maximum Likelihood concatenated phylogeny based on 1860 base pairs of sequence data, from the four combined markers: 16S, COI, H3 and ATPS- $\beta$ . Data is bootstrapped 5000 times and values are noted. An exhaustive search was performed using the TVM+I best fit model. Tree is rooted with *C. nigrolineata*.

## Discussion

Reeb's Pacific *Cellana* phylogeny (figure 1) for the 16S mtDNA marker does not accurately depict the phylogenetic history of *Cellana* species. Low bootstrap values at several branch nodes indicate poor statistical reliability. In addition, a small fragment (270 bp) of the 16S gene was used to create the tree, which Reeb used to infer her conclusions that the Hawaiian *Cellana* colonized the Hawaiian Archipelago multiple times. I tested the validity of Reeb's conclusions by creating phylogenies using multiple

nuclear and mitochondrial markers, including a larger fragment of the 16S ribosomal RNA locus (figures 2-5).

Three out of the four phylogenies oppose Reeb's conclusions, instead supporting Hawaiian *Cellana* monophyly. As expected, my 16S phylogeny topography is similar to Reeb's, however, my phylogeny is statistically reliable, with bootstrap values all greater than 60. The conflicting tree topologies between 16S and the other three markers (COI, H3 and ATPS- $\beta$ ) demonstrate the importance of using multiple markers to accurately illustrate phylogenetic relationships of species assemblages.

The concatenated phylogeny in figure 6 combines data from all four genetic markers and provides solid, statistically reliable evidence for Hawaiian monophyly. In other words, the three Hawaiian *Cellana* species are derived from a common ancestral species that colonized the archipelago once. These findings challenge the conventional assumption that marine species colonize the Hawaiian Archipelago but do not radiate once there.

Given that the monophyletic Hawaiian limpet species occupy a narrow habitat range along the rocky intertidal shores of the isolated Hawaiian Archipelago, this could potentially be an example of sympatric speciation in the marine environment. Four major criteria of sympatric speciation are met in this preliminary study: the most closely related species are found in sympatry, they exhibit reproductive isolation, they are monophyletic, and finally their endemic location in a remote oceanic archipelago makes allopatric differentiation unlikely and less parsimonious than sympatric speciation (Barluenga *et al.* 2006).

The mode of reproductive isolation of sympatric sister species tends to be associated with biological features of organisms as opposed to geographic or distance barriers in allopatric speciation (Coyne and Orr 2004). I can only speculate on the biological mechanism for reproductive isolation of the endemic Hawaiian 'opihi, based on what is known of their reproductive biology. Sexually mature adults spawn, thus, fertilization is external and the larvae develop in the ocean for a three to four days before settling on the shoreline (Kay *et al.* 1982). Spawning of limpets is thought to occur as a result of a combination of environmental cues, one of which is submergence (Iwasaki 1995). Assuming that submergence is an important cue for spawning with the Hawaiian 'opihi, the mechanism of reproductive isolation between them would be timing of reproduction because they each inhabit different zones within the intertidal zone and are submerged at different times. This would allow for the specialization of each Hawaiian *Cellana* species to its own zone within the rocky intertidal shoreline.

In all of the phylogenies presented in this paper (including Reeb's 16S tree), *Cellana sandwicensis* and *C. talcosa* are grouped as sister-taxa. These two species are thus, most closely related and share a most recent common ancestor. Recall that these two species are vertically zoned in the rocky intertidal, such that they are predominantly submerged in deeper areas than *C. exarata*. Being submerged at similar time intervals would provide more opportunity for gene flow between the two species, making them less genetically distinct from each other than from *C. exarata*.

It is consistently shown in the phylogenies (figures 2-6) that the Hawaiian *Cellana* are most closely related to congeners from Japan and the Ogasawara Island chain just south

of Japan. This suggests that the most recent common ancestor of the Hawaiian *Cellana* likely colonized the Hawaiian Archipelago from Japan and/or the Ogasawara Islands.

### **Conclusion**

Until now, evidence for marine radiation within the Hawaiian Archipelago has been poor. This study shows that the endemic Hawaiian ‘opihi are indeed monophyletic. The most recent common ancestor colonized Hawaii and subsequently radiated into the three species we find today. Sequence divergence data from the phylogenies presented in this paper suggest that this ancestor colonized Hawaii roughly around the same time Oahu was formed. Given that the endemic Hawaiian ‘opihi are monophyletic and that they occupy a narrow habitat range in the isolated Hawaiian Archipelago, this may be preliminary evidence for sympatric speciation in the marine environment.

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